Biochimica et Biophysica Acta, 640 (1981) 326—340

BBA 79065

INTERNAL AND EXTERNAL MEMBRANE PROTEINS OF THE CYANOBACTERIUM, *SYNECHOCOCCUS CEDRORUM*

DAVID A. SADEWASSER * and LOUIS A. SHERMAN **

Division of Biological Sciences, University of Missouri, Columbia, MO 65211 (U.S.A.)

(Received August 21st, 1980)

Key words: Iodonaphthyl azide; Lactoperoxidase; Membrane protein; (Photosynthetic membrane)

Summary

The protein composition and architecture of the photosynthetic membranes from the cyanobacterium, *Synechococcus cedrorum*, were analyzed with the aid of site-specific labels. Using membranes labeled with ^{35}S, about 50 membrane proteins can be detected by sodium dodecyl sulfate acrylamide gel electrophoresis. Approximately half of the proteins are accessible to modification by the impermeant probe, lactoperoxidase, indicating that they have surface-exposed domains. At least six of these external proteins can be removed by EDTA washing; the correspondence in molecular weights between five of these EDTA-extractable proteins and those of typical chloroplast coupling factor preparations may indicate that they are subunits of a membrane-bound ATPase. The photoactive, lipophilic compound, [^{125}I]iodonaphthyl azide, was used to label protein domains in contact with the lipid bilayer. Iodonaphthyl azide modification led to a labeling pattern significantly different from that seen with lactoperoxidase. In particular, proteins in the 13 000—20 000 dalton range that were labeled poorly or not at all by lactoperoxidase were heavily modified by iodonaphthyl azide.

Photosystem I and II particles, extracted from the membrane by digitonin treatment, were iodinated by lactoperoxidase after isolation. The PS I particles acted as a relatively tight complex, with most of the proteins remaining inaccessible to surface modification. The PS II particles, on the other hand, responded as a more open structure, with most of the subunits yielding to

* Present Adress: Department of Microbiology, University of California at Los Angeles, Los Angeles, CA 90024, U.S.A.

** To whom correspondence should be addressed.

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; SDS, sodium dodecyl sulfate; Chl, chlorophyll.

lactoperoxidase iodination. Similar studies on a highly fluorescent, temperature-sensitive mutant of *S. cedrorum* revealed a different organization of the PS II complex. This mutant, when grown at 40°C, inserts a 51 kdalton polypeptide in place of a 53 kdalton protein. This protein also replaces the 53 kdalton species in the PS II complex of the mutant after 40°C growth. The structure of this complex is altered in that more sites become accessible to lactoperoxidase. This is particularly true of the 51 kdalton protein, which is barely labeled in wild-type PS II complexes.

Introduction

It is now generally accepted that most of the phospholipid in biological membranes is arranged in a bilayer with the polar head groups at the two surfaces. A more controversial question concerns the exact positioning of the proteins within and around this bilayer. Membrane proteins are divided into two categories: peripheral (or extrinsic) proteins, which are thought to be largely or completely on the aqueous side of the plane defined by the lipid polar head groups; and integral (or intrinsic) proteins, which make substantial contact with the hydrocarbon region of the membrane [1]. It is now clear that most, if not all, peripheral and integral proteins are asymmetrically distributed in the transverse plane of the membrane, and often in the lateral plane as well [2]. Indeed, it is known that even different phospholipid species are asymmetrically distributed in the transverse plane of the membrane [3]. The picture we now have of the membrane is a structure in a constant state of flux. Under physiological conditions, most of the lipids diffuse rather freely in the plane of the membrane and their hydrocarbon chains are disordered (see Ref. 2 for a review of these properties). A number of proteins also seem to diffuse rather rapidly in the lateral plane of the bilayer, a finding that has given rise to the fluid mosaic model of membrane structure [1].

In our study of temperature-sensitive mutants of the cyanobacterium, *Synechococcus cedrorum*, we have found that slight conformational changes in particular proteins can have profound effects on a variety of functions [4,5]. An understanding of such complex interactions requires a much more complete description of the structure of the photosynthetic membrane than is now available. In particular, there is almost no information as to the localization of photosynthetic membrane proteins with respect to the lipid bilayer. Certain studies using electron microscopy, immunological techniques and membrane fractionation have managed to determine the position of a small number of membrane components [5]. However, until recently, no attempt was made to analyze the positions of the majority of proteins, the precise function of which is unknown. This picture is beginning to change as chemical modifiers and site-specific probes are being used to analyze the structure of the photosynthetic membrane [6,7]. Nonetheless, no such work has yet been reported for the photosynthetic membranes of the cyanobacteria. These procaryotes perform an aerobic photosynthesis similar to that of green plants, but possess a number of benefits including ease of genetic manipulation.

This report details the use of a combination of analytical techniques to

probe the protein architecture of cyanobacterial membranes. Three different labeling techniques were used to achieve identification of proteins as (1) associated with the membranes in general, (2) accessible to surface modification, and (3) accessible to internal modification by a hydrophobic nitrene-precursor compound. Growth of algal cultures in media supplied with [^{35}S]-sulfate results in the labeling of all membrane proteins containing cysteine and methionine. This technique provides an estimate of the total abundance of membrane proteins. The second technique, lactoperoxidase-mediated iodination, labels only those proteins having susceptible residues (tyrosines) exposed on membrane surfaces [8]. The third general labeling technique involves the penetration of the lipid-permeant compound, iodonaphthyl azide, which has been shown to attack and covalently bind internal protein domains upon photoactivation [9]. Thus, a comparative analysis of the autoradiographic patterns produced from these different probes would begin to sort out the relative abundance of external and internal proteins.

We are also interested in identifying specific proteins visualized on acrylamide gels with their function. The number of readily identifiable proteins in photosynthetic membranes is quite small, particularly in cyanobacterial membranes. In an effort to assign functional status to some of the proteins in our autoradiograms, we report tentative identification of six peripherally labeled proteins which are extractable by EDTA, a technique commonly used to remove CF_1 from chloroplasts [10]. We also report the lactoperoxidase [^{125}I]iodination of digitonin-treated membrane subparticles [11]. Predictively, treatments which perturb the lipid bilayer by solubilizing adjacent lipid-protein complexes would be expected to expose previously inaccessible moieties to surface modification. We find a correspondence between the [^{125}I]iodonaphthyl azide-labeled internal proteins of 'intact' membranes and some of the bands of the surface-labeled detergent particles. Identification and localization of the proteins associated with the photosystem particles are significant in themselves for an increased understanding of the functional complexes that compose the photosynthetic apparatus.

Materials and Methods

Cell growth and membrane preparation

Synechococcus cedrorum was cultured from stocks of strain UTEX 1191 (University of Texas Culture Collection) in BG-11 medium [12] under vigrous aeration at 30°C. Cultures in late log phase were pelleted by centrifugation (2000 × *g*, 5 min). The pellet was resuspended in a solution of 10 mM KCl, 20 mM Tricine, pH 7.5. and 0.5 M sucrose at a chlorophyll concentration of 300—700 μg Chl/ml. Spheroplasts were prepared by incubating the cell suspension in 2 mg/ml lysozyme (Sigma) with 10 mM EDTA for 2 h at 37°C. The spheroplasts were then broken by Braun homogenization as previously described [11], and broken membranes were collected in the supernatant of a 1500 × *g* spin (5 min). DNAase at 1 μg/ml and 15 mM $MgCl_2$ were added to the membrane suspension which was then sonicated for 2 min. The membranes were collected by centrifugation at 40 000 rev./min for 20 min and the final pellet was resuspended in 10 mM KCl, 20 mM Tricine, pH 7.5.

Toluenesulfonyl flouride (Sigma) was added at a concentration of 1 μg/ml to inhibit proteases. Membranes were stored at −70°C.

Radioactive labeling

^{35}S was incorporated into cell constituents by supplementing the sulfate source of the BG-11 medium with $Na_2{}^{35}SO_4$ at a specific activity of 100 mCi/mM.

Lactoperoxidase-catalyzed iodination of tyrosine and other amino acids in membrane proteins was achieved according to modifications of the method of Hubbard and Cohn [13]. 2 ml of membrane suspensions in pH 7.5 buffer containing less than 500 μg Chl/ml were treated with freshly prepared solutions of lactoperoxidase (1 μg/ml), glucose oxidase (9.1 μg/ml), and glucose, (0.2 mM final concentration). $Na^{125}I$ (New England Nuclear) was added to this suspension to give 10^7 cpm/ml in the mixture. After vortex mixing gently, the sample was incubated at 20°C for 15 min. The mixture was then centrifuged at 30 000 rev./min for 20 min and the pellet was washed twice in fresh suspension buffer. Protein-incorporated radioactivity was measured as trichloroacetic acid-precipitable counts in a Beckman LS230 scintillation counter. Protein was determined by using the method of Lowry et al. [14]. In a control experiment, membranes were iodinated in the presence of 1% digitonin. The results of this labeling were identical to that found in the absence of detergent, indicating that digitonin had no effect on the lactoperoxidase reaction.

In most experiments, a control sample was labeled in the absence of lactoperoxidase. Although this control was generally negative, some experiments showed significant ^{125}I labeling. The gels of such controls had faint bands corresponding to the major bands in the lactoperoxidase-catalyzed iodination. The reasons for such results are summarized in Ref. 8; in our case, it was most likely due to old $Na^{125}I$ and the concomitant formation of I_2, as well as the presence of intrinsic peroxidases. To ensure that the lactoperoxidase-labeled species were indeed external, membranes were also labeled with the impermeant compound, [^{125}I]iodosulfanilic acid (unpublished observations). The results were qualitatively identical to the lactoperoxidase experiments.

[^{125}I]Iodonaphthyl azide was prepared according to the method of Bercovici and Gitler [15] using diaminonaphthalene as the starting compound. Freshly prepared solutions of iodonaphthyl azide (in ethanol) containing $2 \cdot 10^6$ cpm/ml were added to membrane suspensions so as to keep the final ethanol concentration under 1%. Iodonaphthyl azide and treated suspensions were kept away from direct light prior to 310 nm irradiation of 2-ml samples in quartz cuvettes with a monochromatic light source. Irradiation was completed after a 10 min exposure and the succeeding centrifugations and resuspensions were carried out as described above for lactoperoxidase iodination.

Electrophoresis and autoradiography

Radioactively labeled membranes were solubilized in a mixture containing 1.8% SDS, 10% glycerol, 0.05 M Tris-HCl, pH 6.8, and 6% β-mercaptoethanol, and boiled for 1 min. Solubilized proteins were applied to 10—15% acryl-

amide gradient slab gels prepared with the buffer system described by Laemmli [16]. Molecular weight standards were coelectrophoresed as described previously [11].

Slab gels for autoradiographic analysis were dried with a Hoeffer gel dryer for 90 min under a vacuum and exposed to Kodak X-Omat XR-5 X-Ray film at −80°C. Exposures were intensified with Dupont-Cronex Lightning-Plus intensifier screens. Specific exposure times varied among experiments and details are given in figure legends. The detection efficiency of gels loaded with total counts under 500 000 cpm was increased by treating gels with the flourography procedure of Bonner and Laskey [17] or with Enhance (New England Nuclear) prior to drying.

Preparation of submembrane fractions

EDTA extracts were made by suspending lactoperoxidase-[^{125}I]iodinated membranes in 1 mM EDTA, pH 7.5, to a final chlorophyll concentration of 0.1 mg/ml; the mixture was stirred for 15 min at 22°C. The supernatant from a 15 000 rev./min, 5 min centrifugation was collected and concentrated by dialysis against poly(ethylene glycol).

Digitonin-fractionated submembrane particles were prepared as described previously [11], and the photosystem-enriched fractions were separated by sucrose density gradient centrifugation. Subsequent procedures for radioactive labeling of digitonin submembrane fractions were carried out as for unfractionated membranes.

Results

The proportion of membrane proteins that have surface-exposed iodinatable groups was distinguished by differential autoradiography as illustrated in Fig. 1. The left-hand panel shows the lactoperoxidase-iodination pattern of a typical preparation of *S. cedrorum* membranes. The autoradiogram on the right shows the electrophoretic banding pattern of membrane proteins from cultures grown in the presence of [^{35}S]sulfate. We have found empirically that the resolution of individual proteins is much superior on ^{35}S autoradiograms as opposed to Coomassie blue-stained patterns. The latter method has a lower affinity for minor polypeptides, and in order to visualize these, major proteins must be overloaded on the gel resulting in obscuring of bands near the abundant bands in the 18 and 52 kilodalton regions. ^{35}S autoradiograms resolved about 50 distinct bands of which twelve polypeptides are especially prominent in the corresponding lactoperoxidase-iodinated membranes. It is also significant that the intensity of label in corresponding bands between these two methods was not directly proportional to abundance in all cases. For example, the prominent bands in Fig. 1A at 34 and 28 kilodalton were not especially prominent in Fig. 1B.

Autoradiograms of typical wells from SDS-acrylamide slab gels are shown in Fig. 2 comparing: (1) lactoperoxidase-iodinated membranes, (2) EDTA extract from lactoperoxidase-iodinated membranes, and (3) iodonaphthyl azide-labeled membranes. Particularly heavily labeled bands with lactoperoxidase occurred at 72, 56, 50, 46, 34, 32, 28, 15, 13.5, 12.5 and 11.5 kilodal-

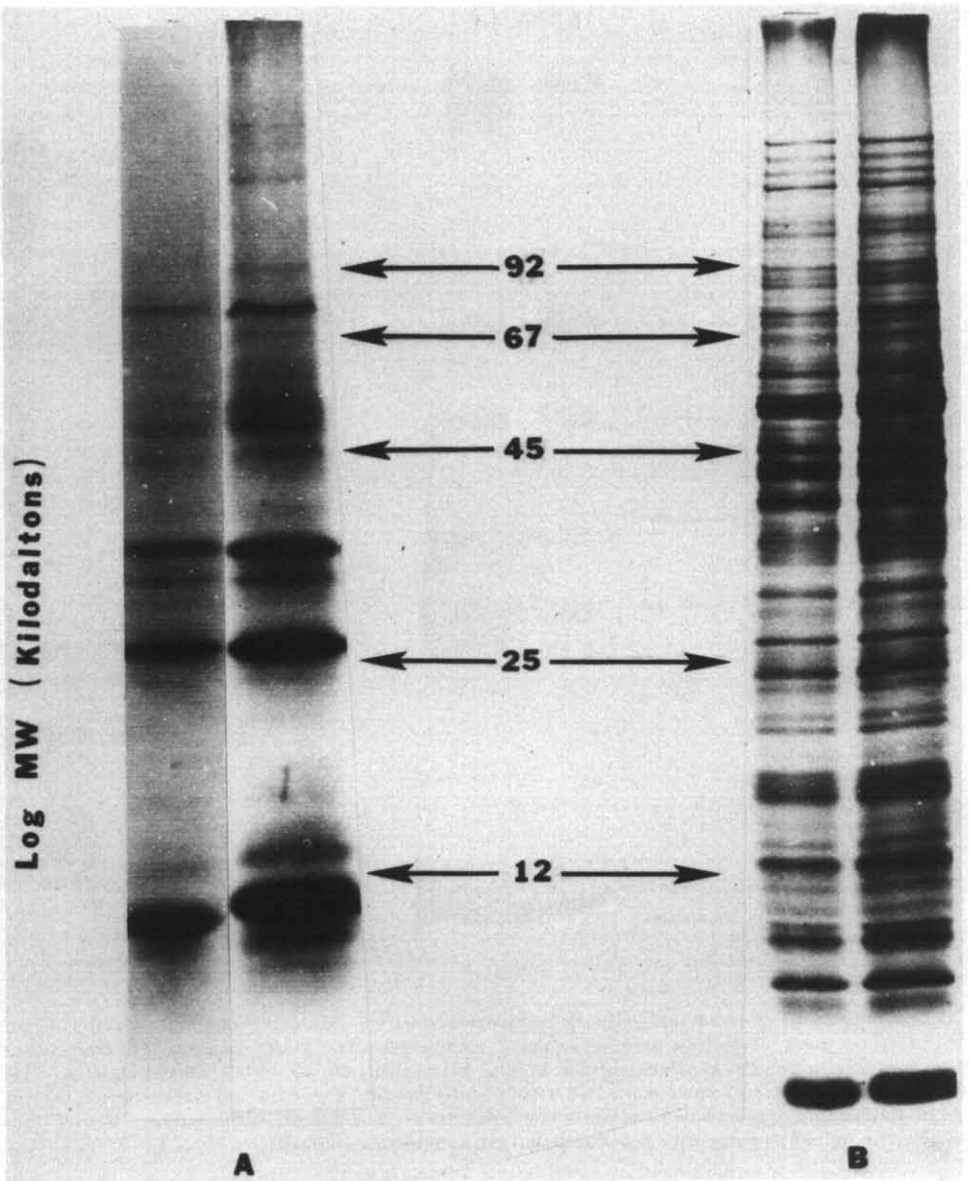

Fig. 1. Autoradiograms of SDS-acrylamide slab gels showing labeled *S. cedrorum* membranes. (A) Membrane labeled with ^{125}I by the lactoperoxidase technique described in Materials and Methods. The two samples contain 40 μg protein and 55 000 cpm ^{125}I (left), and 60 μg protein and 82 000 cpm ^{125}I (right). (B) Membranes labeled with ^{35}S after growth for 24 h in low sulfate growth medium containing 100 mCi/mM ^{35}S. Samples contained 35 μg protein and 50 000 cpm (left), and 45 μg protein and 65 000 cpm (right). Molecular weights were determined by coelectrophoresis of the protein standards: phosphorylase *a*, bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c* [11].

tons. Another 11 bands were distinct but much less prominent. EDTA extracts of these membranes show prominent bands at 72, 56, 50, 36, 28 and 10 kilodaltons. The autoradiogram of iodonaphthyl azide-labeled gels markedly con-

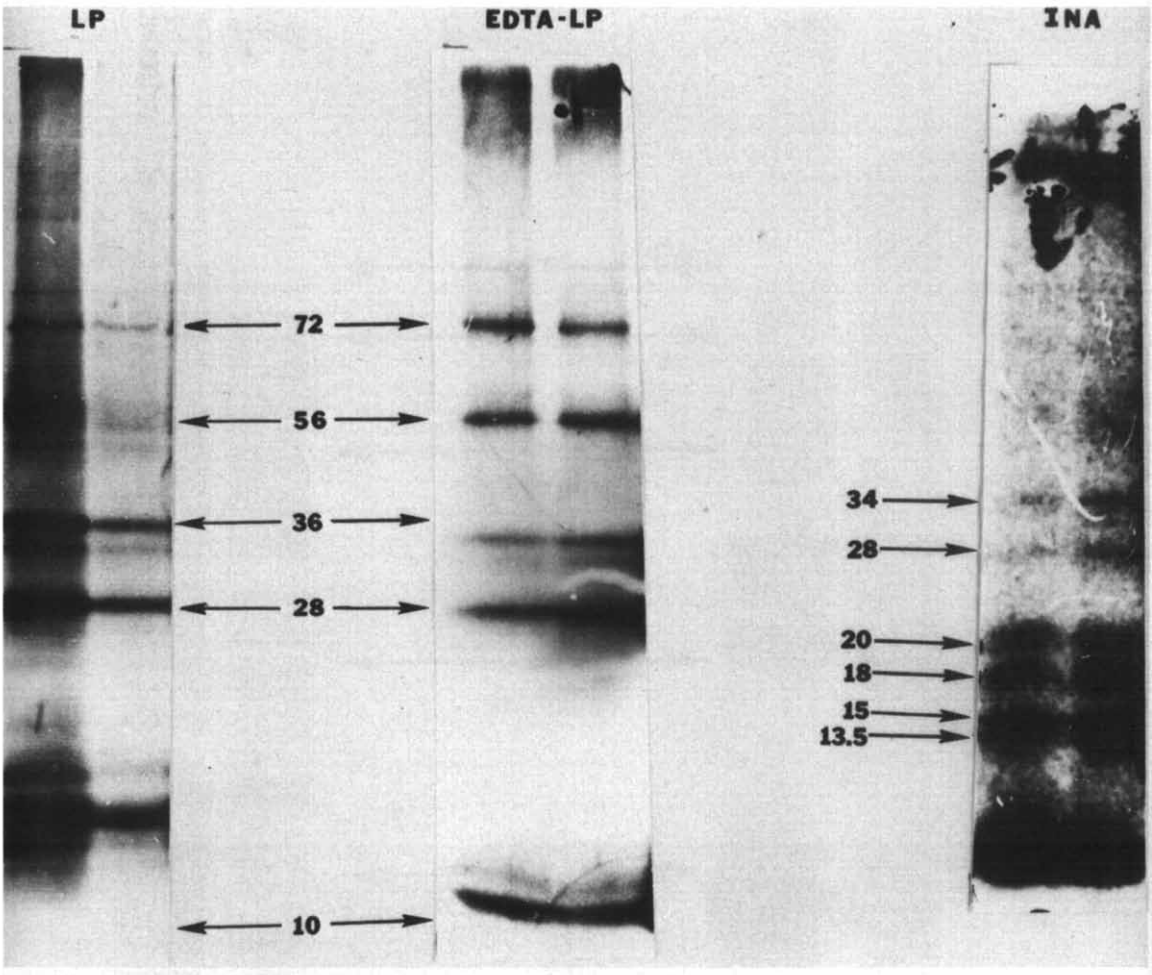

Fig. 2. Autoradiograms of (from left to right): lactoperoxidase-[^{125}I]iodinated membranes (LP), concentrated EDTA extracts of lactoperoxidase-iodinated membranes (EDTA-LP), and [^{125}I]iodonaphthyl azide-labeled membranes (INA). Wells contained (from left to right) 60, 30, 40, 40, 45 and 50 μg protein. Lactoperoxidase-iodinated samples contained 10 000—30 000 cpm/μg protein and were exposed to film for 24 h. Iodonaphthyl azide-labeled samples contained 1000 cpm/μg protein and required 2-week film exposures. The figure is a composite from three slab gels to illustrate comparisons.

trasts with those labeled by lactoperoxidase iodination. In addition to diffuse areas at the origin of the analyzing gel and the dye front (lipid and unincorporated iodonaphthyl azide), major bands are apparent at 34, 28, 20, 18, 15 and 13.5 kilodaltons.

Fig. 3 compares and summarizes the banding pattern of the three autoradiographic conditions shown in Fig. 2. It is important to point out that commonality of bands at a given molecular weight from two different labeling procedures does not automatically mean that a given protein is tagged by both labels. This is because multiple species exist at several molecular weights which are not distinguishable by a one-dimensional technique. Nonetheless, analysis of

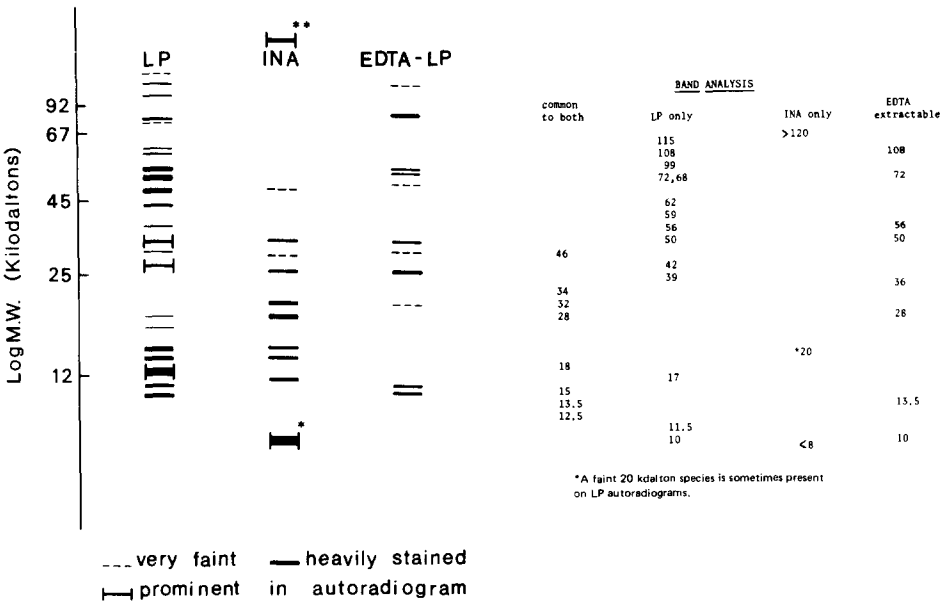

Fig. 3. A summary of the audioradiographic band patterns obtained from membranes electrophoresed as in Fig. 2. The table to the right indicates molecular masses (in kdaltons) of those protein species common to both labeling methods and those peculiar to each. * Lipid and free iodonaphthyl azide (INA); ** high molecular weight or insoluble lipoprotein. LP, lactoperoxidase.

the results depicted in Figs. 1—3 leads to the following conclusions. (1) Most of the proteins that are labeled exclusively by lactoperoxidase have molecular weights above 50 kilodaltons. (2) EDTA extraction removes six of the proteins that are most heavily labeled by lactoperoxidase. (3) A number of polypeptides in the 12—34 kilodalton range were accessible to both probes. (4) Only one protein (20 kilodaltons) was labeled by the permeant probe but not by the impermeant probe. Therefore, the above procedures appear to be capable of resolving proteins that have only external domains, those that have domains externally exposed and buried in the lipid bilayer, and those that are wholly contained within the bilayer.

One of the more important problems in photosynthetic research concerns the location of the proteins that comprise PS I and PS II. The isolation of fractions from *S. cedrorum* enriched in either PS I or PS II has been reported [11], and their protein composition analyzed. The purest PS II particles contain seven proteins (71, 63, 53, 47, 36, 18 and 14 kilodaltons) while PS I particles consist of five polypeptides (61, 18, 17, 15 and 14 kilodaltons). In order to determine the relative position of these proteins in the membrane, the photosystem particles were isolated, iodinated by lactoperoxidase and then analyzed using SDS-acrylamide gel electrophoresis (Fig. 4). In this experiment, the proteins that become accessible to lactoperoxidase iodination can be assessed and compared with the iodination pattern of intact membranes. The photosystem particles seen in Fig. 4 were purified only through the sucrose gradient sedimentation step of the original procedure [11], in order to analyze a larger

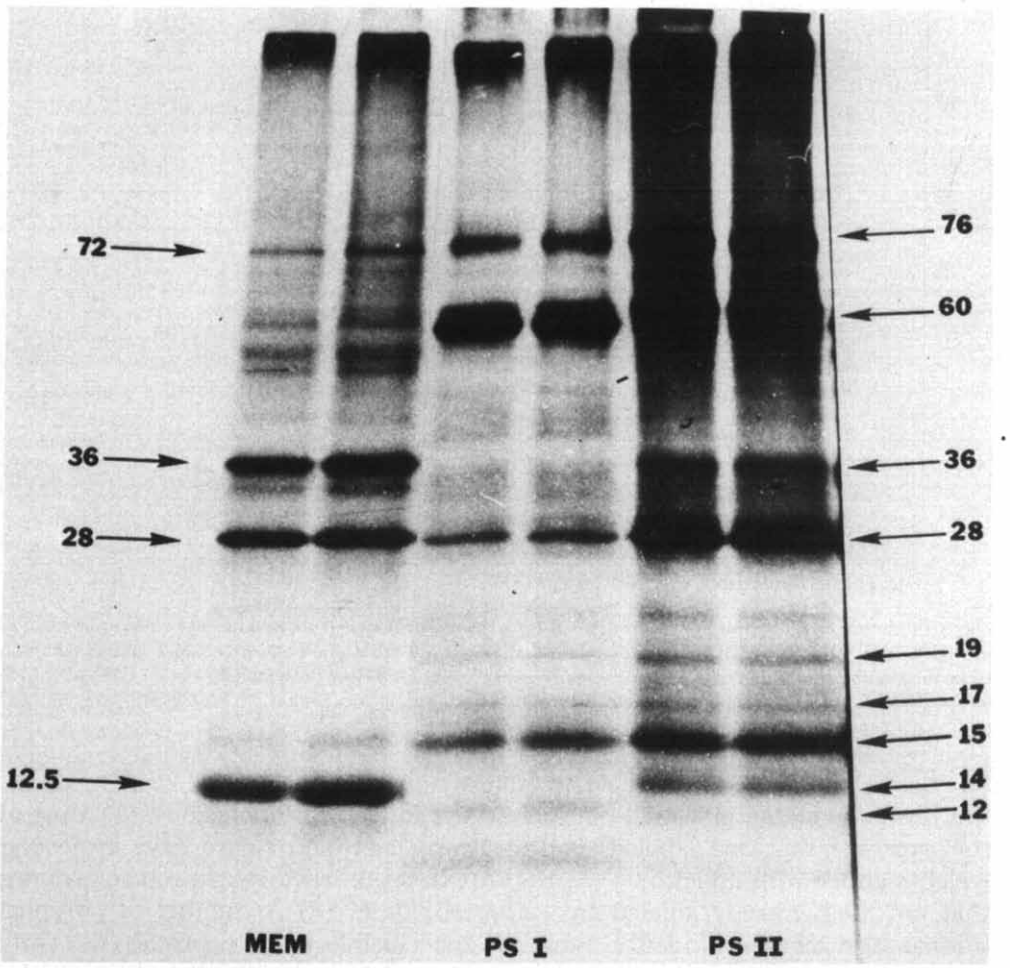

Fig. 4. Composite autoradiogram of (from left to right): control lactoperoxidase-iodinated membranes (MEM), PS I particles, and PS II particles prepared using digitonin as previously described [11]. In each set the left-hand sample contained 75 000 cpm ^{125}I and the right sample 100 000 cpm.

number of proteins. Therefore, there are a few more proteins present than in the purest preparations, particularly in the PS II fraction. Nonetheless, some banding comparisons in this experiment are striking and noteworthy, especially with the pattern of stained proteins in Fig. 7 and Table V of Ref. 11.

The 61 kilodalton protein is a major component of the PS I complex (17% of the total Coomassie blue-staining material) and is labeled extremely heavily by lactoperoxidase. Since there is a band at approximately the same molecular weight which is lightly labeled by lactoperoxidase in whole membranes, it may be that these are the same polypeptide. If so, it implies that the protein normally has some external tyrosines, but that many more are exposed when the PS I particle is removed from the membrane. The very heavy labeling by lactoperoxidase would imply that the 61 kilodalton protein is most likely on the outside of the particle. This is important, because the PS II particle con-

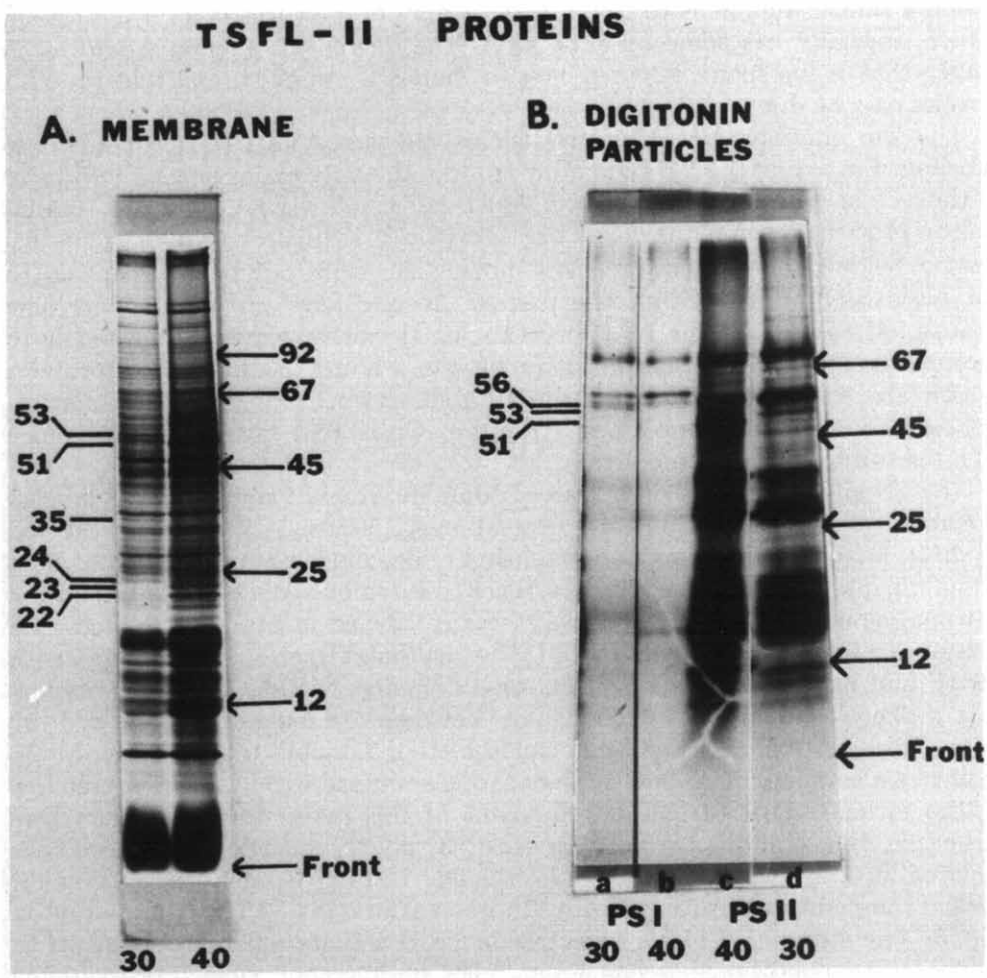

Fig. 5. (A) ^{35}S-labeled membrane proteins of the temperature-sensitive mutant, *S. cedrorum tsfl*-11, grown at either 30 or 40°C. 100 000 cpm per sample. (B) ^{125}I-labeled photosystem particles from the mutant grown at the two temperatures. 75 000 cpm per sample. The protein changes in the 18—25 kdalton range of the ^{35}S-labeled membranes were also found in wild-type membranes used as a control; they are not discussed further.

tained a polypeptide of about 63 kilodaltons which was only present in small quantities and which we thought might represent an artifact. The lactoperoxidase pattern of the PS II particle again shows very heavy labeling in this molecular weight region. One interpretation of these results is that the 61 kilodalton protein is on the outside of the PS I particle and is in close proximity to PS II. During fractionation, this protein can remain attached to some PS II particles; many of these may be removed during chromatography on DEAE cellulose. Similarly, the purest photosystem preparations show a 71 kilodalton protein in the PS II particle but nothing in this molecular weight range in PS I. The labeling pattern in Fig. 4 indicates that lactoperoxidase can label a protein in this range in membranes, as well as in both particles.

Using a similar argument to that above, it may be that this is a PS II protein, which normally has some external sites. The degree of labeling would again imply that it has many exposed sites in the PS II complex, and that the PS I species may be due to contamination.

The two photosystems differ in their overall accessibility to lactoperoxidase labeling. Excluding the 72 kilodalton protein, the only major labeled PS I band is the one at 61 kilodaltons. Of the two other bands visible, only the 15 kilodalton protein is seen in the purest particles (the 28 kilodalton protein will be discussed below). The other three low molecular weight polypeptides appear to be essentially buried within the particle or else have no exposed tyrosine groups. Almost all of the PS II proteins, on the other hand, are accessible to lactoperoxidase; the exceptions may be the 47 and 53 kilodalton proteins which are barely detectable on the autoradiogram. Thus, the PS II particle appears to be a relatively open structure, while PS I gives the appearance of a tight, rather closed complex.

The 28 kilodalton protein is also of some interest. In stained or ^{35}S-labeled membranes, it is present at quite normal levels. However, it is heavily labeled by both lactoperoxidase and iodonaphthyl azide, and is always one of the main contaminants of the photosystem particles (it is removed during DEAE cellulose chromatography). In Fig. 4, it is very heavily labeled in the PS II particle, and is seen as a fairly intense band in PS I. The fact that it fractionates primarily with PS II, and has both internal and external domains, would make this polypeptide a likely candidate for a 'connector' between the reaction centers and the phycobilisomes on the membrane surface. It is possible that such a protein might be a component of the light-harvesting antennae within the membrane.

The ultimate goal for the development of this methodology is to analyze structural defects in the temperature-sensitive, photosynthetic mutants we have isolated in the cyanobacteria [4,5,18]. The results of such an experiment with a temperature-sensitive, highly fluorescent mutant [4,18] is presented in Fig. 5. The strain, *tsfl*-11, is defective in PS II activity when grown at 40°C. Under these conditions, it has a very high fluorescence yield, little variable fluorescence, and almost no 696 nm fluorescence at −196°C. As shown in Fig. 5A, these functional alterations are attended by a change in a membrane protein. The pattern of ^{35}S-labeled proteins shows a decrease in a 53 kilodalton band and a concomitant increase in a 51 kilodalton protein in 40°C membranes compared to 30°C membranes. Thus, the temperature-sensitive properties of *tsfl*-11 appear to be due, at least in part, to a 53 kilodalton → 51 kilodalton alteration in a membrane protein. Since the PS II particles from *S. cedrorum* always contain a 53 kilodalton polypeptide [11], and *tsfl*-11 was defective in PS II activity, we decided to look more closely at the photosystem particles from this strain. The particles were isolated as before and then lactoperoxidase labeled with ^{125}I. As shown in Fig. 5B, the same change is also seen in the PS II protein distribution after lactoperoxidase treatment; a labeled band in the 30°C particles at 53 kilodaltons is replaced by a very heavily labeled band at 51 kilodaltons in the 40°C-grown cells.

There are two other features worthy of note in this experiment. First, the PS I particles from cells grown at either temperature are labeled by lactoperoxidase in an almost identical fashion. (We presume that the 56 kilodalton

protein that appears in the 30°C-grown PS I particles is a contaminant, since it appeared in this experiment only.) Secondly, the lower molecular weight components of PS II are more heavily labeled in both the 30 and 40°C particles of *tsfl*-11 than in the wild-type particles (Fig. 4). It had been noted previously that *tsfl*-11 deviated slightly from the wild-type even when grown at 30°C [4]. The labeling pattern described in Fig. 5 may provide an answer for this phenomenon. The PS II particles from *tsfl*-11 show greater labeling of the 53 kilodalton and low molecular weight proteins at both temperatures; growth at 40°C accentuates this and leads to very heavy labeling of the 51 kilodalton protein. From these observations, we conclude that the PS II particle in *tsfl*-11 is constructed in a more 'open' form than normal. This allows tyrosine groups which are usually buried in the complex to become exposed to lactoperoxidase. The 53 kilodalton → 51 kilodalton alteration at 40°C leads to a PS II particle in which the 51 kilodalton polypeptide has many more exposed tyrosine groups. That this change is indeed due to assembly and not to differences in stoichiometry is emphasized by the fact that the stained patterns indicate a one-to-one exchange of the 51 kilodalton for the 53 kilodalton protein (data not shown).

Discussion

In this report, we have used the site-specific labels, lactoperoxidase and iodonaphthyl azide, to determine the position of proteins in the photosynthetic membrane of the cyanobacterium *S. cedrorum*. The results indicate that approx. 24 proteins have surface-exposed tyrosine groups and that some of these proteins are removed by EDTA washing. It is likely that five of these EDTA-extractable proteins are components of a membrane-bound ATPase complex. A much smaller number of proteins are labeled by the photoactive, hydrophobic probe iodonaphthyl azide; by one-dimensional SDS-acrylamide gel electrophoreses, most of the iodonaphthyl azide-labeled proteins correspond to proteins, also labeled by lactoperoxidase. There is only a protein at 20 kilodaltons which is labeled by iodonaphthyl azide and not by lactoperoxidase. Isolation of photosystem particles and subsequent lactoperoxidase labeling have revealed some important information about the assembly of these structures. PS I appears to be a tightly organized complex with only a 61 kilodalton protein having many exposed tyrosine groups after digitonin extraction. The PS II particle, on the other hand, acts as a much more loosely packed complex, with most proteins being accessible to lactoperoxidase. The structure of the PS II complex is altèred in a temperature-sensitive mutant of *S. cedrorum*, forming a still looser structure. More sites become accessible to lactoperoxidase modification, especially on a 51 kilodalton polypeptide which is normally not significantly exposed in wild-type particles.

The ultimate importance of these conclusions will rest, in part, on the specificity of the probes used. The lactoperoxidase-glucose oxidase system for iodinating exposed tyrosine residues of proteins has proven to be an efficient and specific method for identifying surface-exposed membrane proteins [8]. There is now a great deal of evidence which supports the fact that lactoperoxidase iodination is specific for external protein domains. We have checked

our results with a second impermeant probe, [^{125}I]iodosulfanilic acid, and found the same qualitative pattern. Thus, we believe that the lactoperoxidase labeling gives a true indication of proteins containing surface-exposed domains.

A less well characterized labeling procedure was used to identify proteins containing domains in proximity with the lipid bilayer. Iodanaphthyl azide in the presence of 310 nm radiation is converted to a nitrene capable of covalent attachment with unspecified amino acid residues and lipids. That iodonaphthyl azide partitions into the lipid of membrane suspensions is implied by its high solubility in hexane and low solubility in water [15]. The presumption that only 'internal' proteins are labeled by this compound is supported by the observations of Bercovici and Gitler [15] and Kahane and Gitler [19] and other investigators [20] who also used modified nitrene precursors to label internal regions of simple lipid plus protein vesicles and aggregations. Conrad and Singer [21] have recently questioned the degree to which amphipathic molecules such as iodonaphthyl azide actually intercalate into lipid bilayers, especially those rich in protein such as biological membranes. They suggested that these 'probes' may be forming mixed micelles with the lipids of the membrane on the surfaces of the bilayer. Thus, iodonaphthyl azide may not be washed away by conventional procedures used to remove unincorporated label, but remain inaccessible to internal proteins. If this micellation is occurring in our system, it could explain the unexpectedly low degree of internal labeling we observe with iodonaphthyl azide.

Recently, there has also been some question as to the specificity of labeling hydrophobic domains with nitrenes [9]. Bayley and Knowles [22,23] have determined that nitrenes may be unsatisfactory for such labeling due to their long lifetimes and their electrophilic character. They conclude that nitrenes will eventually lead to preferential labeling of externally located domains. Importantly, they also determined that nitrene labeling was quite inefficient. Though we have not performed all of the controls of Bayley and Knowles, our results would indicate that iodonaphthyl azide may be specific, but it is certainly inefficient. Iodonaphthyl azide labeling leads to a pattern entirely different from that obtained with lactoperoxidase (Fig. 2), when the major iodonaphthyl azide-labeled species are considered (13.5—20 kilodaltons). These proteins are either absent or barely labeled in lactoperoxidase-treated membranes. Nonetheless, when faintly labeled species are considered, the possible non-specificity of iodonaphthyl azide must be taken into account. Thus, the number of proteins thought to contain both internal and external domains might be an overestimate. However, it is the inefficiency of the iodonaphthyl azide labeling which poses the greatest long-range problems. A number of photosystem proteins did not label at all with iodonaphthyl azide, though they were accessible to lactoperoxidase modification after membrane solubilization. An experiment to label first membranes with iodonaphthyl azide and then isolate the photosystem particles was prevented by this low labeling efficiency. Therefore, under the conditions used in our experiments, we believe that iodonaphthyl azide labels protein domains in contact with the lipid bilayer, though not exclusively. It does not appear to be effective, however, in labeling hydrophobic protein domains that are involved in protein-protein interactions.

Despite these uncertanties, the experimental approach that we have described has yielded some important information as to the structure of the photosynthetic membrane. We have seen that of the approx. 50 membrane proteins detectable by one-dimensional SDS-acrylamide electrophoresis, about 24 have sites accessible to lactoperoxidase modification. This is likely to be a lower limit, since some proteins may have either no exposed tyrosine group or may be blocked from acting as a substrate due to steric hindrance. Of these external proteins, at least six heavily labeled species can be removed by an EDTA wash. Based on extensive evidence from a variety of photosynthetic membranes, it is likely that among these proteins removed by EDTA are the subunits for the membrane-bound coupling factor (CF_1 or the ATPase) [10]. The molecular weights of some of these bands are quite similar to those of other well characterized ATPase systems. The reported molecular masses of the F_1 complex from *Escherichia coli* are: $\alpha = 56$ kilodaltons; $\beta = 52$ kilodaltons; $\gamma = 30$ kilodaltons; $\delta = 20$ kilodaltons; and $\epsilon = 10$ kilodaltons [24], while we found EDTA-extractable proteins of 56, 50, 36, 28, 13 and 10 kilodaltons. Recently, Binder and Bachofen [25] isolated the coupling factor complex from a thermophilic cyanobacterium with subunit molecular weights closer to those generally reported from chloroplasts: $\alpha = 59$ kilodaltons, $\beta = 56$ kilodaltons, $\gamma = 37$ kilodaltons, $\delta = 17.5$ kilodaltons, and $\epsilon = 13$ kilodaltons [26]. Their complex had no identifiable δ subunit, and interestingly, this corresponds to a molecular weight region in which we found no major EDTA-extractable species. Since the ATPase complex from *S. cedrorum* has not been characterized, we cannot yet assign a 'coupling factor' label to any of these proteins. Nonetheless, finding the coupling factor on the external surface of the membrane would correspond to the results of other photosynthetic organisms, and such proteins should be labeled by lactoperoxidase.

We have interpreted our labeling of isolated photosystem particles as indicating that PS II forms a looser structure than PS I. This is based on the fact that a larger proportion of the polypeptides are accessible to lactoperoxidase modification. At the present time, it is impossible to assess whether this situation exists in the membrane or was caused by digitonin solubilization. However, the labeling pattern does correspond to the morphology of the particles as observed by electron microscopy [11]. The PS II particles were seen to be short rods with dimensions of 18×6 nm, while PS I occurred as longer rods with an axial ratio of 7 : 1. Thus, PS II particles would tend to have a higher proportion of sites accessible to external modification. Since more of the PS II proteins are available for lactoperoxidase modification in whole membranes (Fig. 3), we can speculate that PS II is normally more externally located than the PS I complex (see also Ref. 11). The results in regard to the location of the 53 kilodalton protein are less ambiguous. In wild-type cells, it is normally buried within the PS II complex. In the mutant *tsfl*-11, however, the protein exists in an environment that is substantially more accessible to lactoperoxidase. It would appear from these observations that the function of PS II can be considerably altered by a slight rearrangement of components in the complex. This structure yields reduced PS II activity, large aberrations in fluorescence induction kinetics, and a near abolition of F696 fluorescence. It will now be of interest to determine which pigments, if any, are normally complexed to this protein.

Despite the high resolution of our SDS-acrylamide gel system, it is still not possible to resolve all protein species by this technique. Therefore, we have begun to analyze membrane structure using the two-dimensional electrophoresis procedure of O'Farrell [27] which separates proteins by both size and charge. We detect nearly 150 spots by this method, and the results presented in Figs. 1—3 have been confirmed by the two-dimensional gels (unpublished observations). However, there is a problem of reproducibility when comparing two or more complex gel patterns, a feature that has been noted by other authors. To solve this problem, McConkey [28] has developed a double-label autoradiography procedure which depends upon coelectrophoresis of two samples and the differential sensitivity of films to isotopes such as ^{3}H and ^{14}C. Since this technique requires an isotope such as ^{3}H, and because of the controversy in regard to nitrene labeling, we are planning to continue these studies with internal probes which generate carbenes and contain ^{3}H. A likely candidate for this work is the compound, [^{3}H]adamantene diazirine, which generates the carbene, [^{3}H]adamantylidene, after photoactivation [29].

Acknowledgements

The authors would like to thank Ms. Jill Cunningham for her capable technical assistance, and Ms. Cathy Huckins for artistic assistance in the preparation of figures. This research was supported by grant GM21827 from the National Institutes of Health.

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